Nicotine-stimulated proteins in mouse cells are distinct from heat-shock proteins

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Treatment of mouse tissue-culture cells with nicotine concentrations of 1 mM or less had no significant effects on cell viability, morphology or protein synthesis, but higher concentrations resulted in both altered cell morphology (rounding and vacuolization) and alterations in [³H]leucine-labelled protein profiles on sodium dodecyl sulphate/polyacrylamide gels. The synthesis of a M_r -70000 protein was increased more than 2-fold relative to that of other major cellular proteins in 3T3 and L929 cells treated with 5 mM-nicotine and in B16 cells treated with 10 mM-nicotine, and this protein appeared to be a soluble cytoplasmic polypeptide. The radiolabelling of several additional polypeptides (M_r 62000 in 3T3 cells, and M_r 45000 and 38000 in B16 cells) was also stimulated by nicotine. The nicotine-enhanced M_r -70000 protein was distinct, however, from a major cell stress/heat-shock protein whose synthesis was stimulated after incubation of cells at 43.5°C for 20 min.

The alkaloid nicotine is a major component of tobacco smoke and is present in much higher concentrations in sidestream smoke (cigarette smoke in the atmosphere of smoke-filled rooms) than in mainstream smoke (cigarette smoke directly inhaled by smokers) (Brunnemann *et al.*, 1978). Nicotine mimics the action of acetylcholine in impulse transmission at mammalian neuromuscular junctions by binding to the nicotinic acetylcholine receptor, a multi-subunit complex of membrane glycoproteins and polypeptides (Conti-Tronconi *et al.*, 1982). There is also evidence for high affinity binding to non-cholinergic nicotine receptors on other cells and tissues, including human phagocytic leucocytes (Davies *et al.*, 1982). The effect of nicotine on the chemotaxis *in vitro* of human polymorphonuclear leucocytes has been studied, and has been found to be both stimulatory (Totti *et al.*, 1984) and inhibitory (Bridges *et al.*, 1977), depending on the concentration of nicotine. Inhibitory effects of nicotine on the reactivity *in vitro* of mouse spleen T and B lymphocytes have also been noted (Roszman, 1977).

Possible effects of nicotine on protein synthesis in cultured cells have not been as well characterized. However, nicotine has been shown to enhance the inhibition by benzo[*a*]pyrene of prolactin and growth-hormone synthesis in cul-

tures of rat pituitary tumour cells (Chakrabarti *et al.*, 1982). The present study was designed to determine if nicotine has any specific effects on cellular protein synthesis that are distinct from general effects on the overall extent of polypeptide synthesis, by using [³H]leucine to label cellular proteins metabolically in several different mouse cell lines treated with various concentrations of nicotine. The identification and partial characterization of a nicotine-enhanced cellular polypeptide are presented here.

Materials and methods

Growth, nicotine treatment and radiolabelling of cells

Mouse L929, 3T3 (BALB/c) and B16-melanoma cell lines were grown as monolayer cultures in Eagle's minimal essential medium (GIBCO) supplemented with 5% foetal-bovine serum (M.A. Bioproducts, Walkersville, MD, U.S.A.) in 25 cm² or 75 cm² tissue-culture flasks. Cultures were used shortly before reaching confluency, and were pre-treated for 4 h with various concentrations of nicotine (Sigma Chemical Co.). Because dilutions were made from a 1 M stock of nicotine in dimethyl sulphoxide, control cultures were similarly pre-treated with corresponding amounts of the latter. Unless stated otherwise, cell cultures were radio-

labelled for 20h in the continuous presence of nicotine, with 10 μ Ci of [4,5- 3 H]leucine (40–60 Ci/mmol; New England Nuclear Corp.)/ml. Equivalent unlabelled cultures were stained with Trypan Blue dye to determine cell viability at the end of the incubation period.

Preparation of subcellular fractions

At the end of the radiolabelling period, cells were harvested by scraping with a rubber policeman into unlabelled minimal essential medium lacking serum, and the cells were pelleted by centrifugation at 1500 rev./min for 2 min. Cells were allowed to swell for 2 min at 0°C in 1.0 ml of E-T buffer (1 mM-EDTA/10 mM-Tris/HCl, pH 7.4) and were then ruptured with 16 strokes of a stainless-steel Dounce homogenizer (clearance 0.005 cm). The cell homogenate was further fractionated into a 'clarified homogenate' (containing soluble cytoplasmic proteins and membrane fragments) and a 'homogenate pellet' (containing unbroken cells, nuclei and large cell debris) by centrifugation for 2 min at 1500 rev./min (Hunt & Summers, 1976). Portions of the clarified homogenate and homogenate pellet (resuspended in 0.5 ml of E-T buffer) from each cell culture were assayed for trichloroacetic acid-insoluble radioactivity (Hunt & Summers, 1976). Particulate and soluble fractions were prepared from the clarified homogenate by centrifugation at 100 000 g for 90 min.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of cellular proteins

Portions of various subcellular fractions from the different nicotine-treated and untreated cell cultures were subjected to electrophoresis in 8% or 10% polyacrylamide slab gels in the discontinuous buffer system of Laemmli (1970). After electrophoresis, the slab gels were fixed and stained in a solution of 0.04% (w/v) Coomassie Brilliant Blue R (Sigma) in 50% (v/v) methanol/7% (v/v) acetic acid. Destaining was done in 5% methanol/7% acetic acid, and the gel was then processed for fluorography (Bonner & Laskey, 1974) and exposed at -70°C with Kodak X-Omat L film. Densitometer scans were made from the developed X-ray films and integrated with a Flur-Vis Auto-scanner (Helena Laboratories, Beaumont, TX, U.S.A.).

A series of [14 C]methylated proteins (New England Nuclear Corp.) were run in the end well of each polyacrylamide slab gel as M_r standards: myosin, 200 000; phosphorylase *b*, 92 500; bovine serum albumin, 68 000; immunoglobulin heavy chain, 50 000; ovalbumin, 43 000; carbonic anhydrase, 29 000.

Results

Effect of nicotine on cell morphology and viability

In the initial experiments, L929 cells were incubated in medium containing nicotine concentrations in the range of 0.1 mM–0.01 μ M, but no apparent effects on cell morphology or cell viability (assayed by Trypan Blue dye exclusion) were observed after 24 h of incubation. However, when L929 or 3T3 cell cultures were incubated with higher concentrations of nicotine (0.2, 1.0 and 5.0 mM), a dramatic morphological change was observed within 2–3 h in the cultures treated with 5.0 mM nicotine (Fig. 1). The cells became more rounded and vacuolated, with the cell rounding more evident with the L929 cells and the vacuolization more pronounced in the 3T3 cells. Also, the nuclei appeared darker with phase contrast and smaller in diameter after nicotine treatment. With the B16 melanoma cells, a similar effect was observed at an even higher nicotine concentration (10 mM rather than 5 mM; bottom panels of Fig. 1).

Cell viability was not significantly affected at these higher nicotine concentrations, and the cells regained their normal morphology within 18 h after re-feeding with fresh medium lacking nicotine. These morphological alterations and their reversibility were very similar to previous observations with chick-embryo fibroblasts infected with a Bryan high-titre strain of Rous sarcoma virus that was temperature-sensitive for transformation (Bader, 1972): when chick cells were switched from a non-permissive (41°C) to a permissive (36°C) temperature, there was a change from an elongated to a polygonal or rounded shape, and a large number of intracellular vacuoles appeared. In addition, transformed chick cells at 36°C became elongated and lost their vacuoles several hours after being shifted to 41°C (Bader, 1972).

Effect of nicotine on cellular protein synthesis

An inhibition of 20% or less of incorporation of [3 H]leucine into cell proteins was observed with L929 and 3T3 cell cultures treated with concentrations of nicotine of 5 mM or lower, but incorporation was decreased to less than 10% of control values after treatment with 10 mM-nicotine. The vast majority of the radiolabel incorporated into proteins (90–95% of the total) was recovered in the clarified homogenate fraction, compared with the homogenate pellet fraction.

As shown in Fig. 2(a) for [3 H]leucine-labelled proteins from the nicotine-treated 3T3 cells, the radiolabelling of two proteins of M_r approx. 70 000 and 62 000 was significantly enhanced in the 5 mM-nicotine-treated cells compared with other cellular proteins of higher and lower M_r values. Compared

Fig. 1. *Morphology of mouse 3T3, L929 and B16 cells before and after treatment with nicotine*
Cells were photographed (phase contrast; magnification $\times 90$) either before the addition of nicotine (—) or approx. 3h after the addition of 5mM-nicotine (3T3, L929) or 10mM-nicotine (B16).

with the radiolabel in a major protein of M_r approx. 55000, that in the M_r -70000 and -62000 polypeptides was increased almost 3-fold in the 5mM-nicotine-treated cells compared with untreated cells, whereas the relative amount of radiolabel in a M_r -43000 polypeptide (presumably actin) was decreased to less than 40% of the control value by the same treatment (Fig. 2b). The metabolic radiolabelling of the same M_r -70000 polypeptide was also significantly increased (more than 2-fold relative to the M_r -55000 and -43000 proteins) in

L929 cells treated with 5mM-nicotine (Fig. 3). However, there was apparently no alteration in the specific radiolabelling of the M_r -62000 and -43000 proteins in these nicotine-treated cells. The M_r -70000 protein from the L929 cells was present in a soluble fraction prepared by ultracentrifugation of the clarified cell homogenate, consistent with this protein being a soluble cytoplasmic polypeptide (results not shown).

In a corresponding experiment with nicotine-treated B16 cells, no inhibition of protein synthesis

Fig. 2. Selective effect of nicotine on the $[^3\text{H}]$ leucine labelling of different protein species in 3T3 cells
 (a) Equivalent portions of clarified homogenates of untreated and nicotine-treated (0.2, 1, 5 mM) cell cultures were electrophoresed in a sodium dodecyl sulphate/polyacrylamide slab gel, and densitometer scans were made of the gel fluorograph. The profiles are shown for the 5 mM-nicotine-treated (A) and untreated (B) cell samples, with the direction of electrophoresis from left to right. The vertical scale is in arbitrary units and normalized with respect to the most intense radiolabelled protein band. The vertical arrows and numbers indicate the positions and M_r values ($\times 10^{-3}$) of the major radiolabelled protein species whose synthesis was either increased (70, 62), decreased (43), or unchanged (55) by the nicotine treatment. (b) The densitometer scans were integrated for the 0, 1 mM- and 5 mM-nicotine-treated cell protein samples, and the relative amounts of radiolabel in the different protein species were plotted, with the values normalized with respect to the major M_r -55000 protein species (■). Other protein species are: M_r 43000 (□); M_r 62000 (○); and M_r 70000 (●).

Fig. 3. Profiles of $[^3\text{H}]$ leucine-labelled proteins from nicotine-treated and untreated L929 cells
 Clarified homogenates of untreated L929 cells (A) and 5 mM-nicotine-treated L929 cells (B) were analysed as described in the legend to Fig. 2(a). The vertical broken line indicates the position of the nicotine-stimulated M_r -70000 protein.

and no significant alterations in the profiles of $[^3\text{H}]$ leucine-labelled proteins were observed with concentrations of nicotine of 5 mM or less (Fig. 4). With 10 mM-nicotine, however, total incorporation of $[^3\text{H}]$ leucine into cell protein was decreased to approx. 40% of the control value, and nearly half of this labelled protein was recovered in the homogenate pellet fraction (as opposed to the 5% or less recovered in this fraction for the 0–5 mM-nicotine-treated B16 cells). This result suggested that the 10 mM-nicotine treatment had altered cell membranes and/or cytoskeletal structure, so that cell breakage by homogenization was less efficient. The incubation with this high concentration of nicotine increased the relative amount of radiolabel in the M_r -70000 protein approximately 2-fold relative to that in the total population of cell proteins and significantly decreased the radiolabel in the M_r -43000 protein in both the clarified homogenate and homogenate pellet fractions (Fig. 4, traces C and D). In addition, a new radiolabelled protein of M_r approx. 38000 was present in the

Fig. 4. Profiles of [^3H]leucine-labelled proteins from nicotine-treated and untreated B16 cells

Portions of clarified homogenates and homogenate pellets of untreated and nicotine-treated (1, 5, 10 mM) B16 cells were analysed as described in the legend to Fig. 2(a). Traces: (A) Clarified homogenate of 0 mM-nicotine-treated cells; (B) clarified homogenate of 5 mM-nicotine-treated cells; (C) clarified homogenate of 10 mM-nicotine-treated cells; (D) homogenate pellet of 10 mM-nicotine-treated cells. The vertical broken lines and numbers indicate the positions and M_r values ($\times 10^{-3}$) of the nicotine-enhanced (70, 45, 38) and nicotine-depressed (43) protein species.

Fig. 5. Profiles of [^3H]leucine-labelled proteins from heat-shock-treated and untreated L929 cells

Portions of clarified homogenates of cells labelled for 2 h after incubation of 0 (A) or 20 min (B) at 43.5°C were analysed as described in the legend to Fig. 2(a). The vertical broken lines indicate the positions of the major heat-shock protein (65) and the nicotine-enhanced protein (70), and the numbers indicate the M_r values ($\times 10^{-3}$) of the major cellular protein species.

clarified homogenate fraction of the 10 mM-nicotine-treated cells (Fig. 4, trace C), and the radiolabelling of a M_r -45 000 protein was also increased compared with that in other major proteins present in the clarified homogenates from the nicotine-treated and untreated cells (Fig. 4, traces A–C).

Mechanism of the nicotine-induced alterations in cellular protein synthesis

Pretreatment of cells with $1\text{ }\mu\text{g}$ of actinomycin D/ml decreased protein synthesis to 10–20% of the control values of L929 cells incubated with 0 or 5 mM-nicotine, and also severely inhibited the nicotine-enhanced synthesis of the M_r -70 000 pro-

tein (results not shown). This result suggested, but did not prove, that a selective increase in the synthesis of mRNA for this protein was occurring in the nicotine-treated cells. Since a similar mechanism has been proposed for cell stress/heat-shock proteins and because heat-shock proteins of M_r approx. 70 000 have been reported for several eukaryotic tissues and cells (Kelley & Schlesinger, 1978; Hightower, 1980), we directly compared the effects of thermal stress and nicotine treatment of L929 cells (Fig. 5). The radiolabelling of a protein of M_r approx. 65 000 was significantly enhanced after the heat-shock treatment, without any significant change in the radiolabelling of the nicotine-enhanced M_r -70 000 protein. Under these conditions of thermal stress, the total protein synthesis was inhibited by less than 10% compared with control cultures. In this same experiment, treatment with 5 mM-nicotine (without heat shock) increased the radiolabelling of the M_r -70 000 protein, but did not affect the radiolabelling of the M_r -65 000 protein (results not shown). Similar thermal stress of the B16 melanoma cells resulted in the increased synthesis of a major heat-shock protein of approx. M_r -66 000, again without a corresponding alteration in the M_r -70 000 protein (results not shown).

We also tested for possible alterations induced by two compounds (pyridine and 1-methyl-2-pyrrolidine) that are analogues of part of the

nicotine molecule in an attempt to understand better the molecular mechanisms of the nicotine-induced alterations. However, these compounds apparently did not enhance or inhibit the effects of nicotine when added in concentrations up to 5 mM, and had no major effects by themselves on cell morphology and protein synthesis in L929 cell cultures (results not shown).

Discussion

We have demonstrated in the present studies with mouse cell cultures that incubation with the same high concentrations of nicotine that induced morphological changes also induced specific alterations in the synthesis of a M_r -70000 cellular polypeptide. The fact that these high, non-physiological, concentrations of nicotine (5 mM or higher) were not significantly toxic to the three different mouse cell lines was surprising, but not totally unexpected, since Bridges *et al.* (1977) had previously reported that the viability of human polymorphonuclear leucocytes fell by only 20% after incubation with 10 mM-nicotine.

Although the identity and function of the major nicotine-induced polypeptide are unknown, this protein is apparently not equivalent to any of the major cell stress/heat-shock proteins described for several different eukaryotic cells (Kelley & Schlesinger, 1978; Hightower, 1980). The synthesis of these particular proteins is stimulated after a variety of stresses, including heat shock, but the function of these proteins is also unknown (Kelley & Schlesinger, 1978; Hightower, 1980). Synthesis of mRNA *de novo* was required for the heat-shock-induced protein alterations (Hightower, 1980), and may also have been required for the nicotine-induced alterations. Changes in protein turnover rate or mRNA stability may also have contributed to the enhanced radiolabelling of the M_r -70000 polypeptide, although the presence of this nicotine-induced alteration in L929 cells after either short (2 h; results not shown) or long (20 h; Fig. 3) labelling periods suggested that increased biosynthesis was primarily responsible for the increased radiolabelling of this protein.

This M_r -70000 protein was not the only protein

whose synthesis was selectively altered in the nicotine-treated 3T3 and B16 cells: we also observed increased radiolabelling of a M_r -62000 protein in the 3T3 cells, increased radiolabelling of M_r -45000 and -38000 proteins in the B16 cells, and significantly decreased radiolabelling of the major M_r -43000 protein in both the 3T3 and B16 cells. Although we did not determine whether the protein-synthesis alterations and the morphological alterations were directly related, the decreased synthesis of the actin-size protein may possibly have affected the cytoskeletal structure and contributed to altered cell morphology in the nicotine-treated cultures.

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References

- Bader, J. P. (1972) *J. Virol.* **10**, 267-276
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83-88
- Bridges, R. B., Kraal, J. H., Huang, L. J. T. & Chancellor, M. B. (1977) *Infect. Immun.* **16**, 240-248
- Brunnemann, K. D., Adams, J. D., Ho, D. P. S. & Hoffman, D. (1978) *Proc. Jt. Conf. Sensing of Environmental Pollutants*, 4th, 876-880
- Chakrabarti, S., Hanes, S. D. & Biswas, D. K. (1982) *Biochem. Biophys. Res. Commun.* **108**, 596-603
- Conti-Tronconi, B. M., Gotti, C. M., Hunkapiller, M. W. & Raftery, M. A. (1982) *Science* **218**, 1227-1229
- Davies, B. D., Hoss, W., Lin, J. P. & Lionetti, F. (1982) *Mol. Cell. Biochem.* **44**, 23-31
- Hightower, L. E. (1980) *J. Cell. Physiol.* **102**, 407-427
- Hunt, L. A. & Summers, D. F. (1976) *J. Virol.* **20**, 637-645
- Kelley, P. M. & Schlesinger, M. J. (1978) *Cell* **15**, 1277-1286
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Roszman, T. L. (1977) in *Pulmonary Disease: Defense Mechanisms and Populations at Risk* (Clark, M. A., ed.), pp. 234-249, Tobacco and Health Research Institute, Lexington
- Totti, N., McCusker, K. T., Campbell, E. J., Griffin, G. L. & Senior, R. M. (1984) *Science* **223**, 169-171